Preparation of Ribonuclease A Surface-Imprinted Nanoparticles with Miniemulsion Polymerization for Protein Recognition in Aqueous Media

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Abstract

Molecularly imprinted polymers represent a new class of materials possessing high selectivity and affinity for the target molecules, offering an array of possible applications in the field of analytical chemistry and separation. In this study, miniemulsion polymerization had been employed to prepare regularly shaped surface-imprinted nanoparticles that displayed affinity towards their template Ribonuclease A in an aqueous media. FESEM, BET and rebinding tests were carried out on the product nanoparticles for morphological and selectivity studies. The nanoparticles exhibited superior loading and selectivity in water, providing a promising material for bioseparation in solid-phase extraction and chromatography.

1.0 Introduction

For many years, enzymes have constituted an inspiration for researching into their synthetic equivalents. Since being pioneered by Wulff[1], molecular imprinting has become an effective way to prepare cross-linked polymer materials that show a 'memory effect' toward their template molecules. To date, molecular imprinting has been successfully applied to small molecules[2-4] through the conventional bulk imprinting approach, whereas much difficulty has been encountered for macromolecules like proteins. With bulk polymerization, many binding sites are embedded deep within the polymeric matrix and this restricts their accessibility to the template macromolecules. On top of that, the traditional imprinting approach has its inherent drawbacks, for example, post-imprinting grinding of the imprinted polymer will give irregularly edgy polymers where their application in chromatographic packing will be limited. In addition, with poor thermal dispersion, bulk polymerization is not suitable to be employed at the industrial scale.

In view of the various limitations of the bulk imprinting, redox-initated miniemulsion polymerization had been applied as an alternative approach for protein imprinting in this work. Miniemulsion polymerization is a polymerization technique that can give regularly shaped polymeric nanoparticles of sizes between 50 and 500 nm. In this way, the quality of the imprinted polymer is ensured. With excellent heat dispersion, this approach will be suitable for large-scale commercial production. On top of that, through the miniemulsion polymerization, imprinted sites were formed on the surface of the imprinted nanoparticles. This is known as surface imprinting and it helped to circumvent the restricted diffusion issue often associated with protein and large molecules. Methyl methacrylate (MMA) and ethylene glycol dimethacrylate (EGDMA) had been used as the functional and cross-linker monomer respectively and the model template protein was Ribonuclease A (RNase A). With the formation of hydrophobic imprinted cavities on the surface, the imprinted nanoparticles displayed high loading and good selectivity towards the template RNase A in water. The realization of the recognition property in water, instead of non-polar media, is essential since it is more similar to a biological system and is thus, more applicable for bioseparation.

2.0 Experimental

The RNase A surface-imprinted nanoparticles (MIP) were prepared using the miniemulsion polymerization modified from Miller's work[5]. The first aqueous phase was prepared by dissolving 0.375 g poly(vinyl alcohol) (PVA), 57.7 mg sodium dodecyl sulfate (SDS) and 46.9 mg sodium bicarbonate in 20 ml of deionised (DI) water. To prepare the

second aqueous phase, 0.2 g of PVA and SDS were dissolved in 400 ml of DI water. Following that, 0.8 ml of MMA and 4.2 ml of EGDMA were mixed, forming the oil phase. The monomer mixture oil phase was then slowly added into the first aqueous phase using a syringe pump (NE-1000 Multi-Phaser, New Era Pump Systems Inc., USA) at a flow rate of 200 µl/min, followed by homogenization at 24,000 rpm with a homogenizer (T25B, Ika Labortechnik, Germany) for 40 seconds. Subsequently, 25.6 mg of the template RNase A was added into the miniemulsion and mixed magnetically for 30 minutes to allow effective monomer-template interaction. The miniemulsion was then added into the second aqueous phase and transferred into a 1 L 3-neck round-bottomed flask. The flask was mechanically stirred (RW20, Ika Labortechnik, Germany), and slowly heated to 40 $^{\circ}$C. When the pre-polymerization miniemulsion reached 40 $^{\circ}$C, the reaction vessel was pre-purged with nitrogen gas for 15 minutes to displace oxygen and finally, sodium bisulfite (0.230 g), followed by ammonium persulfate (0.252 g), were added into the mixture for polymerization up to 24 hours.

Upon completion, the polymeric nanoparticles were washed five times each with DI water and a solution of SDS: acetic acid (10 w/V%: 10 V/V%) to remove the template protein, four times with excess ethanol to remove the surfactant or any unreacted monomer and initiator, and six times with DI water. The washed imprinted polymer was diluted with DI water and kept as a suspension under room temperature. Non-imprinted nanoparticles (NIP) were prepared in a similar manner as above, except without the addition of the template RNase A.

In the morphological characterization of the MIP nanoparticles, field-emission scanning electron microscopy (FESEM) (JSM-6700F, JEOL, USA) and nitrogen sorption method (NOVA 3000 series, Quantachrome Instruments, USA) were used. The swelling ratios of the nanoparticles were also determined. In examining the selectivity of the MIP nanoparticles, batch and competitive rebinding tests were carried out with bovine serum albumin (BSA) as the competitive assay. In addition, the adsorption kinetics was also studied.

3.0 Results and Discussion

As seen in Figure 1, highly monodispersed, regularly shaped RNase A imprinted (MIP) and non-imprinted (NIP) nanoparticles particles that sized around 40 nm were synthesized. The swelling ratios (SR) of NIP and MIP are presented in Table 1. The SR values are direct indications of the extent of cross-linking of the polymeric nanoparticle[6]. In molecular imprinting, highly cross-linked imprinted polymers are usually prepared for effective maintenance of the imprinted sites formed during the imprinting polymerization. The SR values obtained in this work are close to that obtained by Lu et al[6], illustrating that sufficient amount of cross-linking had been achieved. The specific surface areas of NIP and MIP (in Table 1) are not vastly different. However, notably, the values achieved are not as high as that obtained in Vaihinger's work[7] (about 58.0

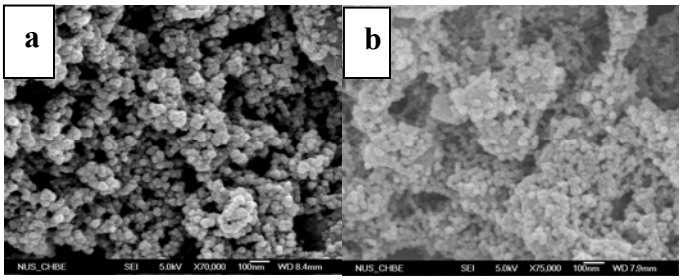

Figure 1. FESEM images of the (a) NIP and (b) MIP nanoparticles.

m^2/g), despite the sizes of the particles in that work (about 200 nm) are much bigger than ours. This could be attributed to the difference in the porosity of the nanoparticles prepared in the respective works. On top of that, agglomeration is often observed for very fine hydrophobic particles at the dried state. When NIP and MIP nanoparticles were lyophilized for BET measurement, it was highly possible that they agglomerated extensively, thus resulting in the smaller measured specific surface area. Nevertheless, although having very large surface could be advantageous for loading and rebinding kinetics, this could in fact compromise the selectivity of the imprinted nanoparticles since non-specific adsoption on the non-imprinted areas would be expected.

Table 1. Morphological feature of the NIP and MIP nanoparticles.

Polymer	Mean diameter (nm)	Swelling ratio	Specific surface area (m^2/g)
NIP	41.0 ± 6.4	4.75 ± 2.08	19.3
MIP	37.6 ± 5.3	7.19 ± 1.22	29.6

The batch rebinding test results are shown in Figure 2. No significant trends were observed for BSA adsorption while in the case of RNase A, the template protein displayed preferential binding to the MIP over the NIP nanoparticles. In addition, the highest RNAse A loading observed for the MIP nanoparticles (at 1.8 mg/ml) was higher (754.0 mg RNase A/g MIP) than that achieved in a similar work by Pang et al[8].

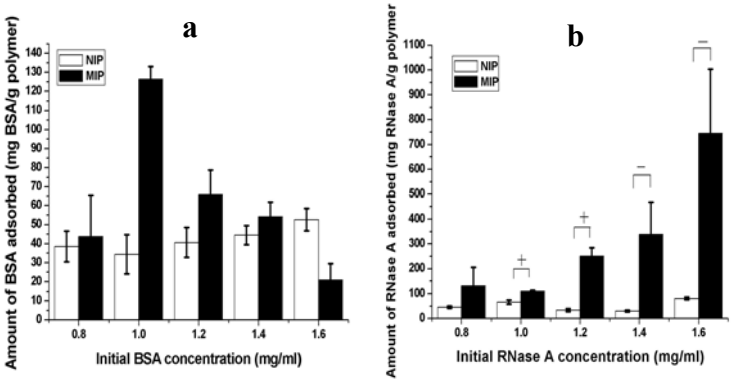

Figure 2. Batch-rebinding tests of (a) BSA, (b) RNAse A for NIP and MIP nanoparticles; Student's t-test, + : $p < 0.05$; - : $p < 0.08$.

For the competitive rebinding test, as shown in Figure 3, a higher BSA loading (26 mg BSA/g NIP) than RNAse A (17 mg RNase A/g NIP) was observed for NIP in a protein mixture. However, when the similar test was conducted for the MIP nanoparticles, comparatively, a marked increase of about 80% in the binding of the template protein to the polymer was observed while the BSA loading (27 mg BSA/g MIP) remained unchanged. It was deduced that the protein loading of NIP is mostly non-specific and the marked increase observed in the adsorption of RNase A to MIP is a strong proof that recognition property had been imparted onto the imprinted nanoparticles. Based on the highest RNase A loadings observed for NIP and MIP nanoparticles, the calculated imprinting efficiency is 9.21.

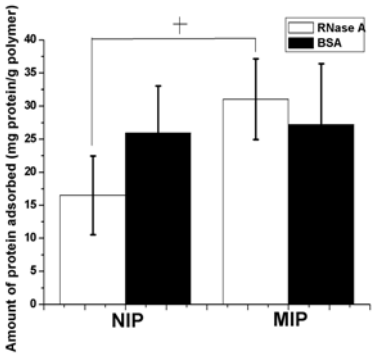

Figure 3. Competitive rebinding tests of RNase A and BSA for the NIP and MIP nanoparticles; Student's t-test, + : $p < 0.12$.

Extensive non-specific adsorption is commonly observed for small particles with very large surface area and this could possibly mask the recognition property of the imprinted nanoparticles[9]. However, in this work, high template loading and good selectivity were detected through the routine rebinding tests in spite of the small sizes and the lower than expected surface areas of the MIP nanoparticles. This indicated that effective imprinting had been achieved and the nanoparticle surface was mostly occupied by imprinted sites for the template RNase A.

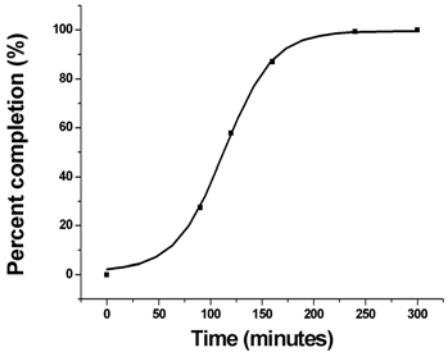

Figure 4. RNase A rebinding kinetics for the MIP nanoaprticles.

In adsorptive separation, the rebinding kinetics will be an important consideration. The kinetics of RNase A adsorption to the MIP nanoparticles was shown in Figure 4. The rebinding process reached its equilibrium in approximately 250 minutes. Given the bulkiness of the protein macromolecules, the rebinding kinetics is rapid and highly desired.

In this study, the mechanism of protein surface imprinting through the miniemulsion polymerization was hypothesized and illustrated in Figure 5. When the template RNase A was added into the pre-polymerization miniemulsion, being amphipathic, the protein molecule was solubilised across the 2 phases separated by micelles. Polymerization reaction was then initiated with the template molecule trapped on the micelle surface. Upon completion and subsequent template removal, binding sites of the complementary protein hydrophobic sections were formed on the nanoparticle surface. During an aqueous rebinding process, protein molecules with the complementary shape to the binding sites would bind preferentially to the imprinted nanoparticles through hydrophobic interaction. Though hydrophobic interaction is less specific compared to covalent, ionic or hydrogen interaction, its ease of application and the applicability in an aqueous medium make it a worthy choice of interaction for molecular imprinting.

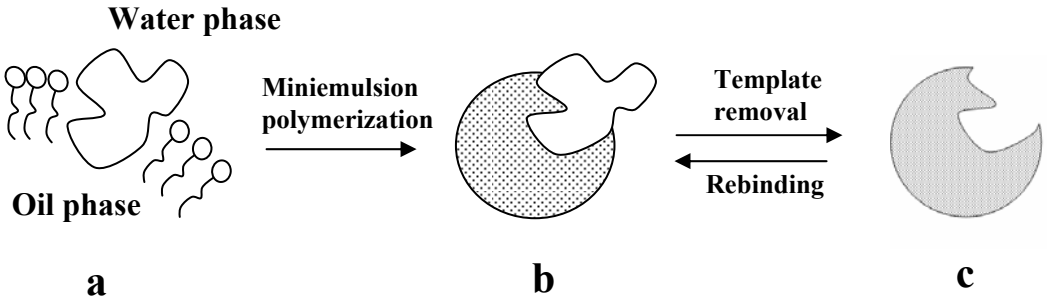

Figure 5. Schematic representation of RNase A surface imprinting through miniemulsion polymerization (a) solubilisation of template RNase A into the micelles; (b) molecular imprinting on the surface of the nanoparticles; (c) removal of the template RNase A molecules frees the imprinted cavities.

4.0 References

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